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Cloning and Characterization of the ε and ζ Isoforms of the 14-3-3 Proteins

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ABSTRACT

Two prominent proteins (30 and 33 kD) in a purified preparation of the sheep pineal gland were studied. Amino acid analysis of tryptic peptides indicated that the 33-kD protein was the ε isoform of the 14-3-3 family of proteins, and that the 30-kD protein was the ζ isoform. The sheep pineal gland was found to have six other 14-3-3 isoforms in addition to the ε and ζ, suggesting that copurification of the ε and ζ forms may reflect the existence of homo- or heterodimers comprised of these isoforms. To characterize 14-3-3 proteins further in the pineal gland, the full sequence of the ε isoform and a partial sequence of the ζ isoform were cloned from a rat pineal cDNA library and are reported here. Tissue distribution studies using Western blot analysis revealed that rat pineal and retina have levels of 14-3-3 protein similar to those found in brain, and that relatively low levels occur in other tissues. This investigation also revealed the ε isoform was present at high levels in the rat pineal gland early in development and decreased steadily thereafter and that 30-kD isoforms exhibited the inverse developmental pattern.

INTRODUCTION

THE 14-3-3 PROTEINS are a family of highly homologous acidic proteins that are widely distributed in nature; highly conserved isoforms have been found in human, rat, sheep, *Xenopus*, *Drosophila*, yeast, and several plants (Aitken *et al.*, 1992). Most 14-3-3 isoforms have an apparent mass of 29-30 kD; the ε isoform is distinctly larger (33 kD). These proteins exist as dimers with an apparent mass of 67 kD (Boston *et al.*, 1982a); however, it has not been established whether the isoforms exist as hetero- or homodimers, and whether preferred pairing occurs.

In mammals, the 14-3-3 family of proteins includes at least eight isoforms, which have been found in a limited number of tissues; neural tissue appears to be somewhat more enriched (Boston *et al.*, 1982b; Ichimura *et al.*, 1988; Isobe *et al.*, 1991). It is estimated that these proteins represent approximately 1% of total brain protein (Boston *et al.*, 1982b). The 14-3-3 proteins have been reported to be localized in neurons (Boston *et al.*, 1982a), and to be axonally transported to nerve terminals in axonal ganglion cells (Erickson and Moore, 1980).

Although a global unifying function of these proteins has not been clearly established, it has been postulated that these proteins are involved in a variety of cell functions (for review, see Aitken *et al.*, 1992). These include the activation of tyrosine and tryptophan hydroxylase in the presence of a Ca^{2+}/calmodulin-dependent protein kinase (Ichimura *et al.*, 1987), inhibition of protein kinase C activity (Toker *et al.*, 1990), and stimulation of calcium-dependent secretion in digitonin-permeabilized adrenal chromaffin cells (Morgan and Burgoyne, 1992). The latter two functions seem to be related to sequence similarity with the family of calcium lipid-binding proteins known as annexins or lipocortins (Toker *et al.*, 1990; Roth *et al.*, 1993). A 14-3-3-like protein present in plants has been demonstrated to be associated with the G-box binding complex, a protein complex that binds to the G-box promoter element of many inducible genes in plants (Lu *et al.*, 1992; de Vetten *et al.*, 1992). The ζ isoform (30 kD) has been reported to

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have phospholipase A_2 activity (Zupan *et al.*, 1992); however, this is controversial.[1] Most recently, it has been demonstrated that the bovine ζ isoform is required for activation of the ADP-ribosyltransferase Exoenzyme S, which is a virulence factor secreted by *Pseudomonas aeruginosa* (Fu *et al.*, 1993).

Our interest in 14-3-3 proteins developed as a serendipitous result of our analysis of a purified preparation of sheep pineal arylalkylamine *N*-acetyltransferase (NAT; EC 2.3.1.87; Klein *et al.*, 1992), an enzyme of central importance to the regulation of the daily rhythm in melatonin synthesis in the pineal gland (Klein, 1985). Analysis of a partially purified sheep pineal NAT preparation revealed two prominent proteins (30 and 33 kD), which were subsequently identified as the ϵ and ζ isoforms of the 14-3-3 proteins, as reported here. The finding of 14-3-3 proteins in the sheep pineal gland was extended by determining what other 14-3-3 isoforms were present. Additionally, we found that 14-3-3 immunoreactivity is easily resolved from NAT activity.

To characterize pineal 14-3-3 proteins further, we cloned the full-length cDNA sequence of the rat ϵ isoform. In addition, the developmental appearance and tissue distribution of these proteins were analyzed in the rat. This revealed that brain-like levels of 14-3-3 proteins occur in the rat retina and pineal gland. Other tissues examined had significantly lower amounts.

MATERIALS AND METHODS

Materials

Sheep pineal glands used for purification were obtained from adult males and females (Dorsett × Rambouillet cross) from the department of Pediatrics of Auckland University (Auckland, New Zealand). Animals had been field maintained. On the day they were killed, they were blindfolded during the afternoon and placed in a light-proof holding pen; they were killed under a dim red light. Rat pineal glands were obtained from daytime-killed rats of mixed age and sex (Zivic-Miller, Allison Park, PA).

The following reagents were used: acrylamide, bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, and Tween-20 (Bio-Rad Laboratories, Richmond, CA); bovine serum albumin (BSA), EDTA, EGTA, 5-bromo 4-chloro 3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), ponceau S, and thimerosal (Sigma, St. Louis, MO); [α-^{35}S]dATP (1,000 Ci/mmole), AuroDye forte, and prestained molecular-weight Rainbow standards (Amersham, Arlington Heights, IL); [^{3}H]acetyl-CoA (3 Ci/mmole) and ^{125}I-labeled Protein A (New England Nuclear, Boston, MA); Immobilon P (Millipore Corp., Bedford, MA); nitrocellulose filters (Schleicher & Schuell, Keene, NH); pBluescript SK II$^+$ (Stratagene, La Jolla, CA); AmplitaqI DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT); Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH); *Eco* RI (New England Biolabs, Beverly, MA); goat anti-mouse IgG and goat anti-rabbit IgG both conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD); unconjugated colloidal gold particles (EY Labs, Inc., San Mateo, CA); 4–20% gradient polyacrylamide gels (Novel Experimental Technology, San Diego, CA); gel-filtration molecular-weight standards (Phamacia LKB Biotechnology Inc., Piscataway, NJ); synthetic peptides conjugated to carrier proteins (University of Notre Dame Bioscience Core Facility, Notre Dame, IN); weak anion-exchange HPLC column (WAX, Mac-Mod Analytical Inc. Chaddsford, PA); size-exclusion HPLC column (TSK 3000, Kratos Corporation, New York, NY); reverse-phase HPLC column (Brownlee RP.300, C_{18}, Applied Biosystems Inc., Foster City, CA); Polytron (Brinkman, Westbury, NY); Centricon-10 units (Amicon, Beverly, MA).

Sepharose-cystamine was synthesized using cyanogen bromide-activated Sepharose (Namboodiri *et al.*, 1987a). Oligonucleotide primers were synthesized on a model 380B DNA synthesizer from Applied Biosystems Inc. (Foster City, CA). The polymerase chain reaction (PCR) was performed using a DNA Pacer from Bellco Biotechnology (Vineland, NJ).

METHODS

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis ($NaDodSO_4$): A published procedure was used (Laemmli, 1970).

NAT Assay: NAT activity was measured in 25 μl of each HPLC fraction by a modification (Namboodiri *et al.*, 1987a) of the procedure of Deguchi and Axelrod (1972).

NAT Purification: A modification of a published procedure was used (Namboodiri *et al.*, 1987a). Groups of 30 sheep pineal glands (50–100 mg wet weight) were homogenized at 4°C (Polytron, one gland/ml 10 m*M* ammonium acetate pH 6.5). This was centrifuged (60 min, 100,000 × *g*) and the supernatant was used for further purification, as described in the legend to Fig. 1.

Preparation of Monoclonal Antiserum 8C3: Monoclonal antibodies were produced against protein which eluted with a peak of *N*-acetyltransferase activity (Fig. 1); pools I–IV from the size-exclusion HPLC column were combined and used for immunization. A BALB/c mouse was injected subcutaneously three times, at 2-week intervals (150 μg of protein/injection) (Moffett and Namboodiri, in preparation). Two weeks after the last injection, the mouse was injected intraperitoneally with 200 μg of protein and the animal was sacrificed 4 days later. The spleen was removed aseptically and used for fusion with myeloma cells. Twelve mouse ascites monoclonal antibodies were isolated subsequently and six of these recognized the purified proteins. These antibodies were screened using a nondenaturing gel. The major portion of the gel was electroblotted for Western blot analysis and a 1-cm lane was analyzed for NAT activity. Six antibodies were found to produce a posi-

[1]Confirmation of these results has not been possible (K. Robinson and A. Aitken, unpublished data; Morgan *et al.*, 1992).

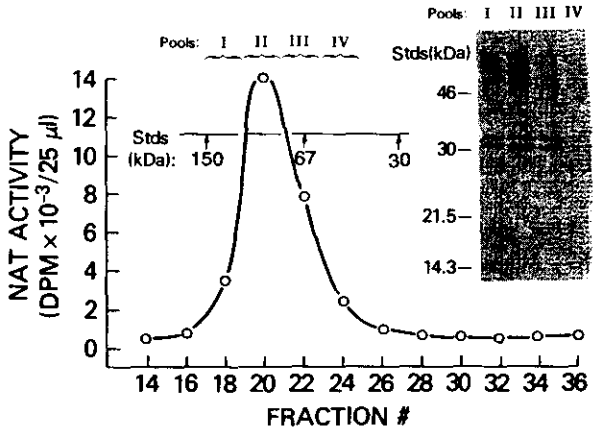


FIG. 1. Purification of sheep NAT by size exclusion HPLC (TSK 3000). Prior to application to the TSK 3000 column, sheep pineal NAT activity was purified as described (Namboodiri *et al.*, 1987a) with some modifications. Pineal cytosol (see Materials and Methods) was applied to a Sepharose-cystamine column. The peak of enzyme activity that eluted after a 12-hr incubation of the column in the presence of 10 m*M* dithiothreitol (DTT) was further purified by HPLC using a weak anion-exchange (WAX) column. The NAT activity was eluted with a NaCl gradient and the late-eluting peak from this step was then applied to a TSK 3000 size-exclusion HPLC column in the presence of 100 m*M* ammonium acetate pH 6.5 and 10 m*M* DTT. The activity eluted as a 100-kD protein as determined by applying molecular-weight standards to the TSK 3000 column under the same conditions (Namboodiri *et al.*, 1987b). The molecular-weight standards are: γ-globulin, 150 kD; bovine serum albumin, 67 kD; and carbonic anhydrase, 30 kD. The peak of activity that eluted at 100 kD was then reapplied to the TSK 3000 column and the resultant elution profile is shown above, with NAT activity measured as described in Materials and Methods. Inset: Pools of fractions were concentrated five-fold by Centricon-10 filtration, separated on $NaDodSO_4$-PAGE (Laemmli, 1970), and transferred to Immobilon P (Towbin *et al.*, 1979). The concentration of protein in the cytosolic preparation was measured prior to electrophoresis by a dye-binding method (Bradford, 1976). To visualize proteins, the blot was blocked with 0.3% Tween in PBS for 45 min followed by incubation with AuroDye forte stain for 2–4 hr. The inset shows two prominent proteins at 30 and 33 kD, and the amount of protein in these two bands correlated with NAT activity.

tive reaction with proteins (30–33 kD) in the fraction containing NAT activity; one (8C3) was also found to be highly specific for the 33-kD protein resolved by $NaDodSO_4$-PAGE.

Preparation of Polyclonal Antisera 274A and 145A: Two synthetic peptides, based on 14-3-3 protein amino acid sequence data that became available during the course of this investigation, were used to generate antisera. Antiserum 274A was generated against a peptide corresponding to a conserved sequence present in the 14-3-3 proteins (F D D A I A E L D T L N E D S Y K D S T L I M Q L L R D N L T L W T S D Q; see Fig. 5, positions 208–244). New Zealand white rabbits, 2–3 kg, were injected intradermally with the peptide at several locations (1 mg on weeks 0, 2, 4, 17, 19, and 21, followed by injection of the colloidal gold conjugated peptide on weeks 41 and 43). Blood was collected from the ear vein on week 45, and serum was stored at −70°C until use. Antiserum 274A reacted with 30- and 33-kD bands of protein, presumably isoforms of the 14-3-3 proteins, according to Western blot analysis of sheep pineal tissue cytosol.

A peptide corresponding to a 33-kD ε isoform sequence (V A G M D V E L T V E E; see Fig. 5, positions 34–47) was used to produce antiserum 145A. The rabbit was injected as described above with purified peptide conjugated to the following ligands: keyhole limpet hemocyanin (weeks 0, 2, and 4), thyroglobin (weeks 6, 8, and 10), colloidal gold (weeks 12, 14, and 16), and bovine serum albumin (weeks 18, 20, and 22). The serum used in the studies presented here was obtained from the week 22 collection. Paradoxically, antiserum 145A did not recognize the 33-kD ε isoform, but instead recognized a 30-kD protein present in sheep pineal cytosol. Antiserum 145A was subsequently affinity-purified using the 30-kD isoforms of the 14-3-3 proteins present in sheep pineal cytosol according to the procedure of Smith and Fisher (1984), with the exception that the antibody was eluted with 0.1 *M* acetic acid pH 2.85 containing 0.1% bovine serum albumin (BSA).

A polyclonal antiserum (Anti-14-3-3) that detected the lower molecular weight isoforms of the 14-3-3 proteins was provided by T. Isobe (Department of Chemistry, Tokyo Metropolitan University, Tokyo, Japan); this antibody was produced against purified bovine brain 14-3-3 proteins as described (Ichimura *et al.*, 1991).

Microsequencing of Proteins: Proteins in the 100-kD fraction from the size-exclusion HPLC (pool II; Fig. 1) were resolved on a 12.5% polyacrylamide gel, transferred onto nitrocellulose, and treated as described (Aebersold *et al.*, 1987). After transfer, proteins were stained with 0.1% ponceau S dye in 1% aqueous acetic acid. Excess stain was removed from the blot by gentle agitation in 1% aqueous acetic acid. Bands of the membrane containing 30- and 33-kD proteins were removed and stored damp-dry at −20°C. Proteins were sequenced using an ABI 477A protein sequencer with 120A online PTH-AA analyzer (Harvard μ-CHEM, Cambridge, MA).

Cloning and Sequencing of Rat ε Isoform of the 14-3-3 Proteins: A cDNA library (817) was prepared from rat pineal poly(A)⁺RNA. The library was constructed in λgt11 *via Eco* RI linker arms as previously described (Huynh *et al.*, 1984) and screened using standard procedures (Sambrook *et al.*, 1989). Briefly, the filters were blocked with 0.3% Tween-20 in phosphate-buffered saline (2.7 m*M* Na_2HPO_4, 1.6 m*M* KH_2PO_4, 154 m*M* NaCl pH 7.2; PBS) for 45 min and incubated with 8C3 antiserum diluted 1:1,000 for 14 hr at room temperature. After washing with 20 m*M* Tris-HCl pH 7.5 at 25°C, containing 500 m*M* NaCl, and 0.05% Tween-20 (TTBS), the filters were incubated with goat anti-mouse antiserum (1:1,000) conjugated to alkaline phosphatase. The immunoreaction was visual-

ized by a chromogenic method with 0.2 mg/ml NBT and 0.2 mg/ml BCIP. Two positive clones were detected; both had 1.7-kb inserts as determined by *Eco* RI excision. Subsequent sequence analysis was performed on one of these clones.

After subcloning the insert into pBluescriptII SK$^+$, the DNA was sequenced by the dideoxy (Sanger *et al.*, 1977) method using the Sequenase Version 2.0 kit according to the manufacturer's protocol for double-stranded sequencing. Several oligonucleotides were chemically synthesized and used as sequencing primers. The strategy for the sequencing is diagramed in Fig. 2.

Cloning and Sequencing of Rat ζ Isoform of 14-3-3: The rat pineal cDNA library was probed with the immunopurified 145A antiserum (1:100) by the same procedure used in the isolation of the ε clone. This resulted in the identification of two positive clones. Of these, one was plaque-purified and determined to be 1.6 kb in length by *Eco* RI excision of the insert. Sequencing template was prepared by the PCR with primers that flank the *Eco* RI cloning site of λgt11. The resultant product was used as template for asymmetric PCR (Medori *et al.*, 1992) to produce single-stranded DNA for the sequencing reaction. The PCR product was then sequenced using the Sequenase Version 2.0 kit following the manufacturer's protocol for single-strand template. The location of the sequencing primers was as described in Fig. 2.

Computer Analysis of Sequences: Sequence analysis was performed using the following programs from the Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984): FASTA, BESTFIT, and PILEUP for homology comparisons of the DNA and protein sequences and PEPTIDESTRUCTURE for predicting protein secondary structure.

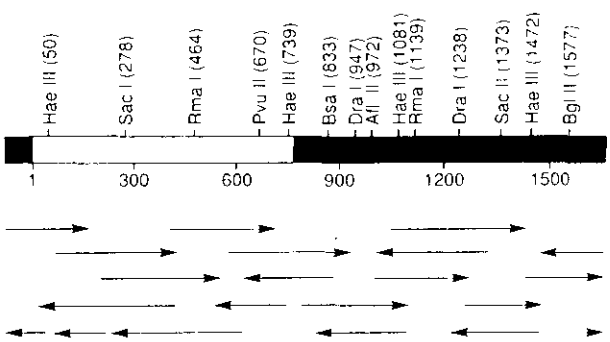


FIG. 2. Sequencing strategy and restriction enzyme map of the cDNA for the ε isoform of the rat 14-3-3 proteins. The open box represents the coding region. The arrows indicated the direction and the region of sequence determined with each primer. The base positions where the various restriction enzymes cut are included in parentheses.

RESULTS

Microsequence analysis of NAT-enriched preparation of sheep pineal gland cytosol

A highly enriched preparation of sheep NAT activity was obtained following size exclusion HPLC using 100 m*M* ammonium acetate buffer pH 6.5 (Fig. 1); the majority of activity eluted in a single peak corresponding to a protein of approximately 100 kD. The four pools of fractions containing NAT activity were analyzed by NaDodSO$_4$-PAGE. Several bands, ranging in size from 30 to approximately 70 kD were present; two prominent bands (30 and 33 kD, Fig. 1, inset) were microsequenced.

33-kD Band: Microsequence analysis of a tryptic digest of the 33-kD protein band yielded sequence from five peptides. These pineal sequences were identical to those found in a partial sequence of the sheep brain 14-3-3 ε obtained from direct sequencing of purified protein (Toker *et al.*, 1992). The sequences of these tryptic peptides are underlined in Fig. 3.

30-kD Band: Analysis of a tryptic digest of the 30-kD band yielded sequences from four peptides (Fig. 4). These sequences showed the highest homology to those found in the sequence of sheep brain 14-3-3 ζ obtained from direct sequencing of purified protein (Toker *et al.*, 1992), with 46 out of 48 amino acids being identical (Fig. 4).

The indications that the 33-kD band was 14-3-3 ε and the 30-kD band was 14-3-3 ζ were extended with efforts to clone cDNA encoding these proteins.

Cloning of 14-3-3 ε from a rat pineal cDNA library

Rat pineal λgt11 (817) and sheep pineal λgt10 libraries were probed. Efforts to screen 80,000 recombinants in the rat pineal cDNA expression library using the 33-kD-selective monoclonal antiserum 8C3 were successful. Two positive clones were identified, one of which was plaque-purified and determined to contain a 1.7-kb insert. Sequence analysis (Fig. 2) indicated this clone (Rat1433ε) contained an open reading frame that coded for a 255-amino-acid protein with a 70-bp ~′ untranslated region and a 900-bp 3′ untranslated region that contained a polyadenylation signal (Fig. 3). The sections of the deduced amino acid sequence of Rat1433ε that correspond to the five tryptic peptides obtained from microsequencing of the 33-kD protein purified from sheep pineal are underlined in Fig. 3. Minor differences were identified between the deduced amino acid sequence of the Rat1433ε and the available sequences obtained by direct analysis of sheep brain 14-3-3 ε peptides (data not shown). These differences occurred at ends of the tryptic peptides, positions that are more likely to be provide unreliable data.

The ε isoform contains 51 acidic amino acids (glutamate and aspartate) and 32 basic amino acids (lysine and arginine), giving it a predicted pI of 4.46. This acidic nature is in agreement with the previously reported acidic nature of the bovine η isoform (Ichimura *et al.*, 1988) and the acidic

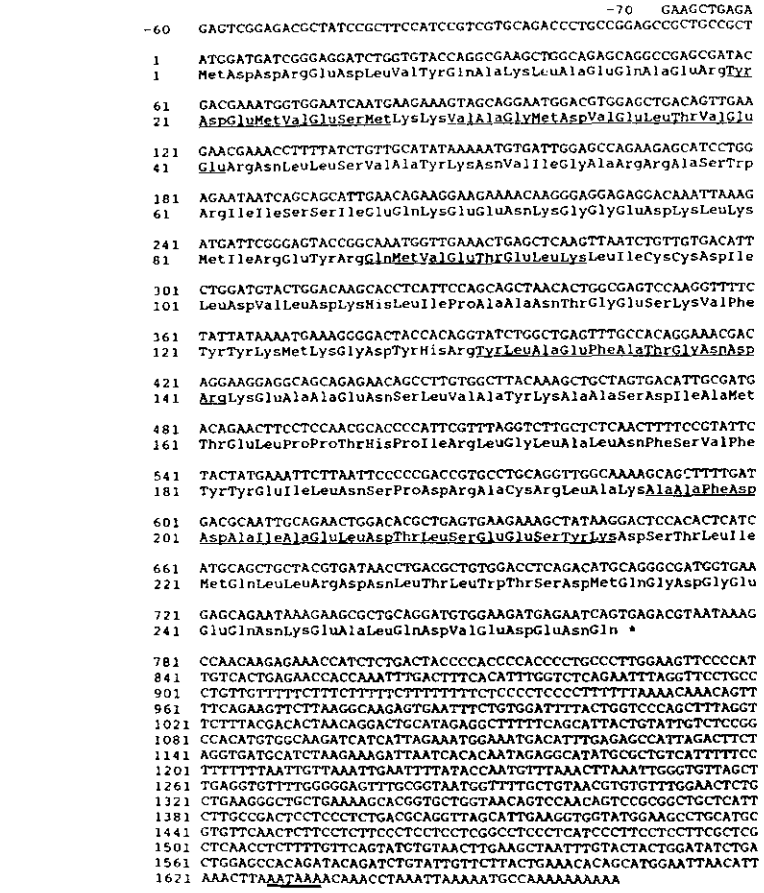


FIG. 3. Complete nucleotide sequence of the cDNA corresponding to the rat pineal ϵ isoform of the 14-3-3 proteins (Rat1433ϵ). The nucleotide sequence was obtained with the strategy diagramed in Fig. 2, and is numbered in the 5′ to 3′ direction with the initiation codon (ATG) as position 1. The deduced amino acid sequence is shown below the nucleotide sequence; (*) denotes the termination codon. The amino acid sequences determined by analysis of tryptic peptides obtained from the 33-kD protein present in an NAT-enriched preparation of sheep pineal cytosol are underlined, and the polyadenylation signal is highlighted by a double underline. This sequence has been submitted to the GenBank/EMBL Data Bank with accession number M84416.

behavior of 14-3-3 proteins in two-dimensional gel electrophoresis (Moore and Perez, 1967). In addition, the carboxy-terminal one-third of the ϵ isoform (positions 172–255, Fig. 3) includes more acidic than basic amino acids resulting in a net negative charge (−13) in this portion of the molecule, a feature that is also seen in the η isoform (Ichimura *et al.*, 1988).

The deduced amino acid sequence of the rat ϵ isoform is identical to that of a mouse 14-3-3 protein (J.E. McConnell, J.F. Armstrong, and J.B.L. Bard, unpublished data; GenBank/EMBL Data Bank accession number Z19599). The cDNA sequences are 98% homologous with differences occurring throughout the molecules. Comparison of the deduced amino acid sequence of the rat ϵ isoform to that of other rat isoforms reveals that the ϵ isoform has

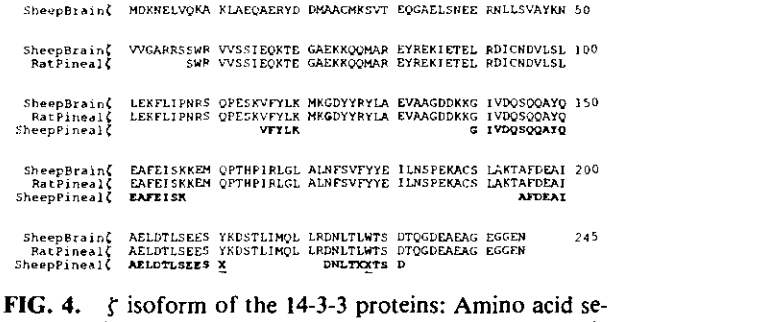


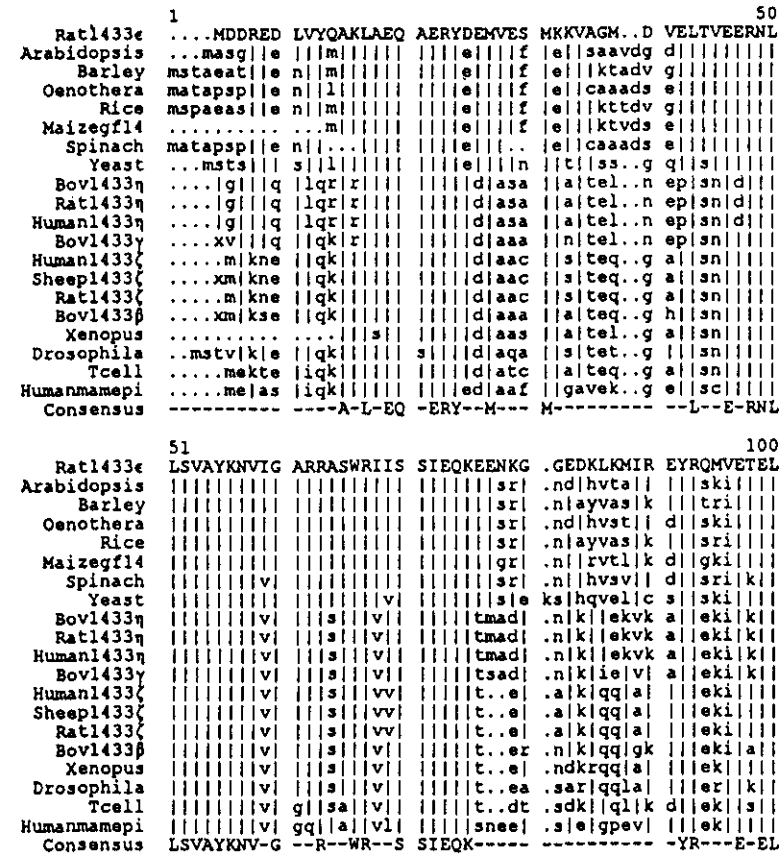
FIG. 4. ζ isoform of the 14-3-3 proteins: Amino acid sequence alignment between the published sequence, tryptic peptides, and the partial-length clone. The alignment contains the amino acid sequence of the ζ isoform obtained from sequencing purified sheep brain 14-3-3 protein (SheepBrainζ; Toker *et al.*, 1992) and the deduced amino acid sequence of the partial-length clone obtained from the rat pineal gland (RatPinealζ). The DNA sequence of the RatPinealζ has been submitted to the GenBank/EMBL Data Bank with accession number L07913. Shown in bold are the amino acid sequences of tryptic peptides obtained from the 30-kD protein present in a purified preparation of sheep pineal NAT (SheepPinealζ). X, discrepancies between the sequence of the sheep pineal tryptic peptides and the published sheep 14-3-3 ζ sequence (see Results section); X, unidentified amino acid.

63% identity and 75% similarity to the η isoform (Watanabe *et al.*, 1991) and 67% identity and 79% similarity to the ζ isoform (G. Rosenfeld, personal communication). Comparison of the nucleotide sequences in the coding region reveals that the ϵ is 66% and 65% homologous to the η and ζ rat isoforms, respectively, and that differences are distributed throughout the molecules.

Cloning of the rat pineal 14-3-3 ζ

A 1.6-kb cone (RatPinealζ) was isolated from rat pineal cDNA library 817 using antiserum 145A, which selectively reacts with the 30-kD protein. Sequence analysis reveals the entire deduced amino acid sequence of the clone (Fig. 4) is identical to a region of the previously published sequence for sheep brain 14-3-3 ζ obtained from direct sequencing of purified protein (Toker *et al.*, 1992). The pineal clone is partial-length and contains 80% of the coding region, beginning at position 58 in the sheep brain ζ sequence and proceeding through the carboxyl terminus (Fig. 4).

The deduced amino acid sequence of the RatPinealζ contains the sequences of all four peptides obtained by microsequencing of the 30-kD sheep pineal protein (Fig. 4). Discrepancies were detected in the sequence of two of the sheep pineal tryptic peptides (X, Fig. 4). Microsequencing identified the amino acids corresponding to positions 211 and 228 in Fig. 4 as alanine and glutamate, respectively. The corresponding amino acids are tyrosine and tryptophan in the published sequence of the sheep 14-3-3 ζ obtained from brain (Fig. 4), in the deduced amino acid sequence of the RatPinealζ (Fig. 4), and in the sequences of other 14-3-3 proteins (Fig. 5, positions 223 and 240). The


1 50
Rat1433ε ....MDDRED LVYQAKLAEQ AERYDEMVES MKKVAGM..D VELTVEERNL
Arabidopsis ...masg||e |||m|||||| ||||e||||f |e||saavdg d|||||||||
Barley mstaeat||e n||m|||||| ||||e||||f |e|||ktadv g|||||||||
Oenothera matapsp||e n||l|||||| ||||e||||f |e||caaads e|||||||||
Rice mspaeas||e n||m|||||| ||||e||||f |e|||kttdv g|||||||||
Maizegf14 ....m|||||| ||||e||||f |e|||ktvds e|||||||||
Spinach matapsp||e n||...|||| ||||e|||.. |e||caaads e|||||||||
Yeast ...msts||| s||l|||||| ||||e||||n |||t||ss..g q|||s||||||
Bov1433η ....|g|||q |lqr|r|||| |||||d|asa ||a|tel..n ep|sn|d|||
Rat1433η ....|g|||q |lqr|r|||| |||||d|asa ||a|tel..n ep|sn|d|||
Human1433η ....|g|||q |lqr|r|||| |||||d|asa ||a|tel..n ep|sn|d|||
Bov1433γ ....xv|||q ||qk|r|||| |||||d|aaa ||n|tel..n ep|sn|||||
Human1433ζ .....m|kne ||qk|||||| ||||d|aac ||s|teq..g a||sn|||||
Sheep1433ζ ....xm|kne ||qk|||||| ||||d|aac ||s|teq..g a||sn|||||
Rat1433ζ .....m|kne ||qk|||||| ||||d|aac ||s|teq..g a||sn|||||
Bov1433β ....xm|kse ||qk|||||| ||||d|aaa ||a|teq..g h||sn|||||
Xenopus ....|||s|| ||||d|aas ||a|tel..g a||sn|||||
Drosophila ..mstv|k|e ||qk|||||| s||||d|aqa ||s|tet..g |||sn|||||
Tcell .....mekte ||qk|||||| ||||d|atc ||a|teq..g a||sn|||||
Humanmamepi .....me|as ||qk|||||| ||||ed|aaf ||gavek..g e||sc|||||
Consensus ---------- ----A-L-EQ -ERY--M--- M--------- --L--E-RNL

51 100
Rat1433ε LSVAYKNVIG ARRASWRIIS SIEQKEENKG .GEDKLKMIR EYRQMVETEL
Arabidopsis |||||||||| |||||||||| ||||||sr| .nd|hvta|| |||ski||||
Barley |||||||||| |||||||||| ||||||sr| .n|ayvas|k |||tri||||
Oenothera |||||||||| |||||||||| ||||||sr| .nd|hvst|| d||ski||||
Rice |||||||||| |||||||||| ||||||sr| .n|ayvas|k |||sri||||
Maizegf14 |||||||||| |||||||||| ||||||gr| .n||rvtl|k d||gki||||
Spinach |||||||v|| |||||||||| ||||||sr| .n||hvsv|| d||sri|k||
Yeast |||||||||| |||||||v|| ||||||s|e ks|hqvel|c s||ski||||
Bov1433η |||||||v|| |||s|||v|| |||||tmad| .n|k||ekvk a||eki|k||
Rat1433η |||||||v|| |||s|||v|| |||||tmad| .n|k||ekvk a||eki|k||
Human1433η |||||||v|| |||s|||v|| |||||tmad| .n|k||ekvk a||eki|k||
Bov1433γ |||||||v|| |||s|||v|| |||||tsad| .n|k|ie|v| a||eki|k||
Human1433ζ |||||||v|| |||s|||vv| |||||t..e| .a|k|qq|a| |||eki||||
Sheep1433ζ |||||||v|| |||s|||vv| |||||t..e| .a|k|qq|a| |||eki||||
Rat1433ζ |||||||v|| |||s|||vv| |||||t..e| .a|k|qq|a| |||eki||||
Bov1433β |||||||v|| |||s|||v|| |||||t..er .n|k|qq|gk |||eki|a||
Xenopus |||||||v|| |||s|||v|| |||||t..e| .ndkrqq|a| |||ek|||||
Drosophila |||||||v|| |||s|||v|| |||||t..ea .sar|qqla| |||er||k||
Tcell |||||||v|| g||sa||v|| |||||t..dt .sdk||ql|k d||ek||s||
Humanmamepi |||||||v|| gq||a||vl| |||||snee| .s|e|gpev| |||ek|||||
Consensus LSVAYKNV-G --R--WR--S SIEQK----- ---------- -YR---E-EL

101 150
Rat1433ε KLICCDILDV LDKHLIPAAN ..TGESKVFY YKMKGDYHRY LAEFATGNDR
Arabidopsis sg||dg||kl ||sr|||||a ..s|d|||||| l|||||||| ||||k||qe|
Barley sk||dg||kl ||s||v|s|t ..aa|||||| l|||||||| ||||ka|ae|
Oenothera sn||gg||kl ||sr|||s|a ..s|d|||||| l|||||||| ||||k||ae|
Rice sk||dg||kl ||s||v|s|t ..aa|||||| l|||||||| ||||ks|ae|
Maizegf14 tk||dg||kl |es||v|sst ..ape||||| l||||||y|| ||||k||ae|
Spinach sdn|dg||kl ||tk|v|||s ..s|d|||||| l|||||||| ||||k||aq|
Yeast tk|sd|||s| ||s|||s|t ..|||||||| ||||||||h| ||||ss|da|
Bov1433η etv|n|v|al |||f||knc| dfqy|||||| l||||||y|| |||v|s|ekk
Rat1433η etv|n|v|al |||f||knc| dfqy|||||| l||||||y|| |||v|s|ekk
Human1433η etv|n|v|sl |||f||knc| dfqy|||||| l||||||y|| |||v|s|ekk
Bov1433γ eav|q|v|sl ||ny||kncs etqi|||||| l||||||y|| |||v|||ek|
Human1433ζ rd||n|v|sl |e|f|||n|s ..qa|||||| l||||||y|| |||v|a|d|k
Sheep1433ζ rd||n|v|sl |e|f|||n|s ..qa|||||| l||||||y|| |||v|a|d|k
Rat1433ζ rd||n|v|sl |e|f|||nrs ..qp|||||| l||||||y|| |||v|a|d|k
Bov1433β qd||n|v|ql |||t|||n|t ..qp|||||| l||||||f|t |s|v|s|dnk
Xenopus qd||k|v|||l ||rf|v|n|t ..pp|||||| l||||||y|| |s|v|s|dsk
Drosophila re||yev|gl |||y|||k|s ..np|||||| l||||||y|| |||v|||da|
Tcell rs||ttv|el |||y||an|t ..np|||||| l||||||f|| |||v|c|d||
Humanmamepi qgv|dtv|gl |||s|||ke|g ..da||r|||h l||||||y|| |||v|||d|k
Consensus -------L-- L---L----- -----S-VF- -KMKGDY--- L-E---G---

151 200
Rat1433ε KEAAENSLVA YKAASDIAMT ELPPTHPIRL GLALNFSVFY YEILNSPDRA
Arabidopsis |d|||ht|a| ||s|q|||na ||a|||||| |||||||y|| ||||||||||
Barley |||||t|||| ||s|q|||la d||t|||||| |||||||||| ||||||||||
Oenothera |||||st|s| ||||q|||na ||a|||||| |||||||||| ||||||||||
Rice |||||t|||| ||s|q|||la d||t|||||| |||||l|||| ||||||||||
Maizegf14 |d||||tm|| ||||q|||la ||a|||||| |||||||||| ||||||||||
Spinach ||||st|t| ||||q|||na ||a|||||| |||||||||| ||||||||||
Yeast ek|tna||e| ||t||e||t| d|||||||| |||||||||| |||q||||k|
Bov1433η nsvv|a|ea| ||e|fe|ske hmq|||||| |||||||||| |||q|a|eq|
Rat1433η nsvv|a|ea| ||e|fe|ske hmq|||||| |||||||||| |||q|a|eq|
Human1433η nsvv|s|ea| ||e|fe|ske qmq|||||| |||||||||| |||q|a|eq|
Bov1433γ atvv|s|ek| |se|he|ske hmq|||||| |||||y|||| |||q|a|eq|
Human1433ζ |givdq|qq| |qe|fe|skk |mq|||||| |||||||||| |||||||ek|
Sheep1433ζ |givdq|qq| |qe|fe|skk |mq|||||| |||||||||| |||||||ek|
Rat1433ζ |givdq|qq| |qe|fe|skk |mq|||||| |||||||||| |||||||ek|
Bov1433β qttvs||qq| |qe|fe|skk |mq|||||| |||||||||| |||||||ek|
Xenopus q|tvas|qq| |qe|fe|sks |mq|||||| |||||||||| |||||||ek|
Drosophila ntvvdd|qt| |qd|f||skg kmq|||||| |||||||||| |||||||||k|
Tcell |qtid||qq| |qe|f||skk |ma|||||| |||||||||| |||||n|el|
Humanmamepi |riidsars| |qe|m||skk |m||n|||| |||||||||h |||a|||ee|
Consensus ---------A Y--A--I--- ----T-PIRL GLALN-S-F- YEI-N-P--A

201 250
Rat1433ε CRLAKAAFDD AIAELDTLSE ESYKDSTLIM QLLRDNLTLW TSDMQGDGEE
Arabidopsis |n|||q|||e |||||||g| |||||||||| |||||||||| |||||d|aad
Barley |n|||q|||e ||||||s|g| |||||||||| |||||||||| |||naee|gd
Oenothera |n||ne|||e |||||||e| |||||||||| |||||||||| |||||d||gd
Rice |n|||q|||| |||||||g| |||||||||| |||||||||| |||nae||gd
Maizegf14 |s|||q|||e ||s|||||| |||||||||| ||||h|||| |||ise|pa|
Spinach |n|||q||ve |||||||g| d|||||||| |||||||||| |||||deaad
Yeast |h|r|q|||| |||||||||| |||||||||| |||||||||| |||ses|qa
Bov1433η |l|||q|||| |||||||n| d|||||||| |||||||||| |||q|deeag
Rat1433η |l|||q|||| |||||||n| d|||||||| |||||||||| |||q|deeag
Human1433η |l|||q|||| |||||||n| d|||||||| |||||||||| |||q|deeag
Bov1433γ |h|||t|||| |||||||n| d|||||||| |||||||||| |||q|d|dgg
Human1433ζ |s|||t|||e |||||||||| |||||||||| |||||||||| |||t|||ea|
Sheep1433ζ |s|||t|||e |||||||||| |||||||||| |||||||||| |||t|||ea|
Rat1433ζ |s|||t|||e |||||||||| |||||||||| |||||||||| |||t|||ea|
Bov1433β |s|||t|||e |||||||n| |||||||||| |||||||||| ||en|||egd
Xenopus |s|||s|||e ||r||||n| |||||||||| |||||||||| ||en||eead
Drosophila |q|||q|||| |||||||n| d|||||||| |||||||||| |||t|||ea|
Tcell |t|||t|||e |||||||n| d|||||||| |||||||||| |||sa|eecd
Humanmamepi is|||tt||e |m|d|h|||| d|||||||| |||||||||| |a|na|eegg
Consensus --L----F-- A---L--L-E -SYKDSTLIM QLL-DNLTLW T---------

251 275
Rat1433ε QNKEALQDVE DENQ..... .....
Arabidopsis ei|||aapkp t|e|q..... .....
Barley ei|||askp| g|gh..... .....
Oenothera ei|||apkpd eqy..... .....
Rice ei|||akpeg egh..... .....
Maizegf14 eir||pkhdl s|g|..... .....
Spinach eit|eaakqq kavnnnkiay .....
Yeast edqqqq|qhq qqqppaaaev khqsk
Bov1433η egn..... ....
Rat1433η egn..... ....
Human1433η egn..... ....
Bov1433γ egn..... ....
Human1433ζ ageggen... ....
Sheep1433ζ ageggen... ....
Rat1433ζ ageggen... ....
Bov1433β agegen.... ....
Xenopus nvegdn.... ....
Drosophila pqeggdn... ....
Tcell aaeg|en... ....
Humanmamepi evpqep|s.. ....
Consensus ---------- ---------- -----

two amino acids in question were probably misassigned during microsequencing because of the weak signals generated by these peptides. Therefore, it is most likely that the sequences of the 30-kD sheep pineal protein tryptic peptides represent 14-3-3 ζ; however, based on the present data, we cannot eliminate the possibility that the tryptic peptides obtained from the sheep pineal gland reflect another 14-3-3 protein isoform.

During the course of this study, we became aware of the nucleotide sequence of a full-length cDNA clone encoding rat brain 14-3-3 ζ (G. Rosenfeld, personal communication). Comparison of the nucleotide sequence of this clone to that of RatPinealζ reveals that, in addition to containing 80% of the coding region, the RatPinealζ clone also contains 93% of the 3′ noncoding region (data not shown). The nucleotide sequences are identical in the coding region; however, there are at least six differences scattered throughout the noncoding region; the basis and significance of which are not clear.

Sequence homology

Figure 5 aligns the amino acid sequences of several isoforms of the 14-3-3 proteins that have been either cloned or directly sequenced from various sources.

Resolution of sheep 14-3-3 proteins and NAT activity as a function of buffer composition

The chromatographic behavior of NAT in size exclusion chromatography is influenced by the eluting buffer. Activity elutes as a 100-kD protein using 100 m*M* ammonium acetate, but as a 30-kD protein when using 100 m*M* sodium citrate (Namboodiri *et al.*, 1987b). This behavior was used to determine if 14-3-3 proteins and NAT activity could be resolved. A crude preparation of sheep pineal glands was applied to a TSK 3000 column; the eluting buffer was sodium citrate (Fig. 6). NAT activity eluted as a 30-kD protein whereas 14-3-3 immunoreactivity (antiserum 274A) was detected in fractions that eluted with a molecular weight of 60–70 kD, fractions in which NAT activity was not detectable (inset, Fig. 6). This indicates that 14-3-3 proteins do not have NAT activity.

Analysis of 14-3-3 isoforms in sheep pineal homogenate

The finding that all five peptides analyzed from the 33-kD band represent the 14-3-3 ϵ isoform indicates this protein represents the major, if not sole, 33-kD protein in this preparation. Similarly, the finding that all four tryptic peptides from the 30-kD band represent the ζ isoform also indicates that this is the major, if not sole, 30-kD protein present in this preparation. One explanation of this observation is that the ζ isoform is the only 30-kD 14-3-3 protein in the pineal gland.

To investigate this, 14-3-3 proteins were purified from the sheep pineal gland using established methods (see legend to Fig. 7). This revealed that the ϵ and ζ isoforms do not dominate but that the α, β, δ, and η isoforms are also present; the γ isoform may be absent (Fig. 7). An unidentified peak (?), which elutes prior to the ϵ, does not appear to correspond to any previously identified 14-3-3 protein isoform in sheep brain. The α, β, δ, and ϵ isoforms are relatively more abundant than the other isoforms in the pineal gland. The ζ and unidentified peak are present at 40% of the level of the dominant isoforms and the η is barely detectable. This demonstrates that the ζ is not the only 30-kD isoform of the 14-3-3 proteins present in the pineal.

FIG. 5. Comparison of the amino acid sequences of various 14-3-3 related proteins to the Rat1433ϵ. The homology comparison was generated using the PILEUP program contained in the GCG sequence analysis package (Devereux *et al.*, 1984). The (|) represents identity with the rat ϵ sequence, and the (.) represents gaps introduced by the computer program to improve alignment. The x in the bovine β and γ and the sheep ζ sequences indicates the amino-terminal residues were *N*-acetylated (Toker *et al.*, 1992). The sequence of the Rat1433ϵ is reported in Fig. 4.[2] The following sequences were compared to the ϵ isoform: *Arabidopsis thaliana* GF14 (Lu *et al.*, 1992); barley (Brandt *et al.*, 1992); *Oenothera hookeri* (Hirsch *et al.*, 1992); rice (Kidou *et al.*, 1993); maize GF14 (de Vetten *et al.*, 1992); spinach (Hirsch *et al.*, 1992); yeast (van Heusden *et al.*, 1992); bovine η (Ichimura *et al.*, 1988)[3]; rat η (Watanabe *et al.*, 1991); human η (Ichimura-Ohshima *et al.*, 1992); bovine γ (Isobe *et al.*, 1991); human ζ (Zupan *et al.*, 1992); sheep ζ (Toker *et al.*, 1992); rat ζ (G. Rosenfeld, personal communication); bovine β (Isobe *et al.*, 1991); *Xenopus laevis* (Martens *et al.*, 1992); *Drosophila* (Swanson and Ganguly, 1992); human T cell (Nielsen, 1991); human mammary gland epithelial cell, human mamepi, (Prasad *et al.*, 1992).

[2]PCR was used to amplify sheep pineal cDNA with primers from the carboxy- and amino-terminal regions of the ϵ protein. A single PCR product (700 bp) was generated; the product was sequenced and it represented 90% of the coding region. The deduced amino acid sequence of the PCR product was identical to that of the rat ϵ isoform presented in this figure. The DNA sequence of the sheep PCR product has been submitted to the GenBank/EMBL Data Bank with accession number L07914.

[3]The carboxy-terminal 45-amino-acid portion of the deduced sequence of bovine 14-3-3 η is identical to the deduced amino acid sequence of an open reading frame contained in an 893-bp sheep pineal cDNA clone. The nucleotide sequence of the clone is 100% homologous to bovine 14-3-3 η in the 135-bp coding region and is 97.7% homologous in the 3′ noncoding region (C.T. Bond, J.L. Weller, P.H. Roseboom, M.A.A. Namboodiri, D.C. Klein, and J. Adelman, unpublished data; GenBank/EMBL Data Bank accession number L07915).

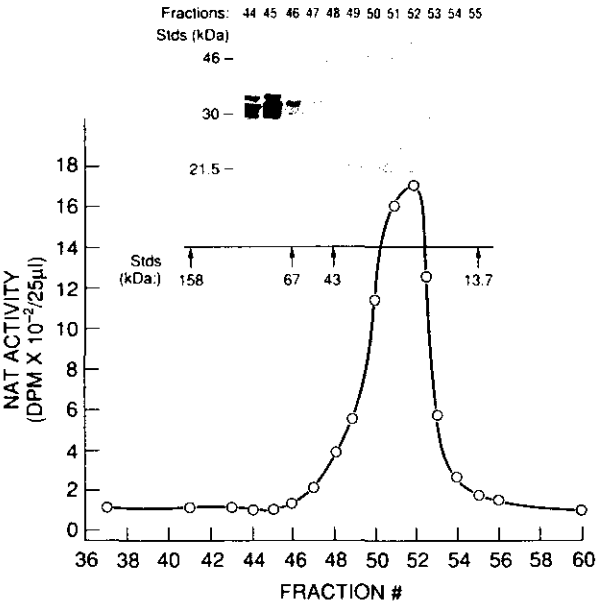


FIG. 6. Size exclusion HPLC analysis in the presence of sodium citrate. Sheep pineal cytosol was prepared by glass/Teflon homogenization (100 mg wet weight/1 ml) in 100 m*M* sodium citrate pH 6.5, 1 µg/ml BSA, and 1 m*M* DTT. The supernatant from a 60-min, 100,000 × *g* spin (1.5 ml) was applied directly to the TSK 3000 HPLC column that had been equilibrated with homogenization buffer. Fractions (3 ml) were collected and NAT activity was measured on 25-µl aliquots as described in Materials and Methods. The molecular-weight standards were passed through the column under the same conditions used for the cytosol. The molecular-weight standards are: aldolase, 158 kD; bovine serum albumin, 67 kD; ovalbumin, 43 kD; and RNAase A, 13.7 kD. Inset: Fractions were prepared for Western blot analysis as described in the legend to Fig. 1. To detect the ε and 30-kD isoforms of the 14-3-3 proteins, the blot was incubated with 274A antiserum (1:1,000) for 18 hr. After washing with TTBS, the blot was then incubated with goat anti-rabbit antibody (1:1,000) conjugated to alkaline phosphatase, and immunoreactivity was visualized by a chromogenic method using BCIP and NBT.

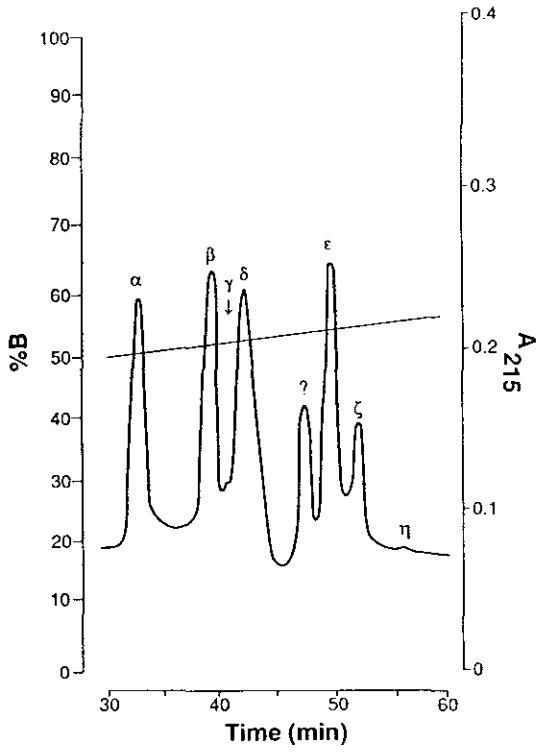


FIG. 7. Elution profile of the 14-3-3 protein isoforms present in sheep pineal cytosol. Sheep pineal 14-3-3 proteins were purified from cytosol as previously described (Toker *et al.*, 1990). The purified protein was applied to a reverse-phase HPLC column using a step gradient with heptafluorobutyric acid as the ion suppressant (Toker *et al.*, 1992). The elution pattern is shown above, and the identity of the isoforms is noted above each of the peaks. The predicted location of the γ isoform, based on analysis of sheep brain 14-3-3 proteins, is noted with (↓); the pineal also contained a peak (?) that was not present in brain.

Developmental expression of 14-3-3 proteins in the rat pineal gland

The developmental pattern of expression of 14-3-3 proteins in the rat pineal gland was studied by Western blot analysis (Fig. 8). A 33-kD band revealed using 8C3 antiserum, presumably the 14-3-3 ε isoform, was present at all times. The levels were highest at day E19 and decreased steadily during development. Analysis with Anti-14-3-3 antiserum, which is selective for the smaller isoforms, revealed that a 30-kD band was detected in all samples; however, the levels were lowest at day E19 and increased steadily throughout development.

Comparative tissue distribution

Western blot analysis of various rat tissues revealed a wide range of concentrations (Fig. 9). The relative abundance of the ε isoform was: cerebellum, cortex, hypothalamus, retina > testis > pineal >>> kidney, spleen > adrenal, lung, heart, liver, ovary, and thyroid. The relative abundance of the 30-kD isoforms was similar; however, slightly higher levels were detected in the cortex, pineal, adrenal, and retina as compared to the ε isoform.

The rat pineal gland contained moderate levels of 14-3-3 protein immunoreactivity. However, the highest concentrations were found in brain tissues, which agrees with previously reported studies measuring levels of the 30-kD 14-3-3 mRNAs or immunoreactivity in human (Boston *et al.*, 1982a), bovine (Ichimura *et al.*, 1991; Isobe *et al.*,

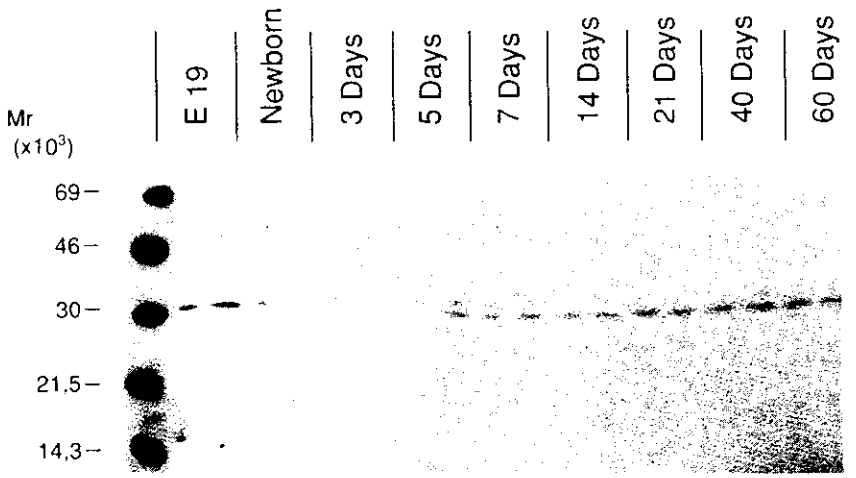


FIG. 8. Immunoblot analysis of the 30- and 33-kD isoforms of the rat 14-3-3 proteins at various stages of development. Cytosol from rat pineal glands obtained on embryonic day 19 (E19), newborn (NB), and at various days after birth were prepared by sonication in 10 volumes (vol/wt) of buffer as previously described (Babila *et al.*, 1992). Samples were run in duplicate, and 30 μg of protein was loaded per well. Proteins were resolved by NaDodSO$_4$-PAGE and transferred to an Immobilon P membrane as described in the legend to Fig. 1. The blot was blocked with 0.3% Tween-20 in PBS for 30 min and incubated with Anti-14-3-3 antiserum (1:2,000) for 18 hr at room temperature. After washing, the blots were incubated with goat anti-rabbit antiserum (1:1,000). The immunoreactivity was visualized with ^{125}I-labeled protein A (100,000 cpm/ml) followed by autoradiography. The ε isoform of the 14-3-3 proteins was detected by repeating the above procedure using the 8C3 monoclonal antibody (1:2,000) and goat anti-mouse antibody (1:1,000).

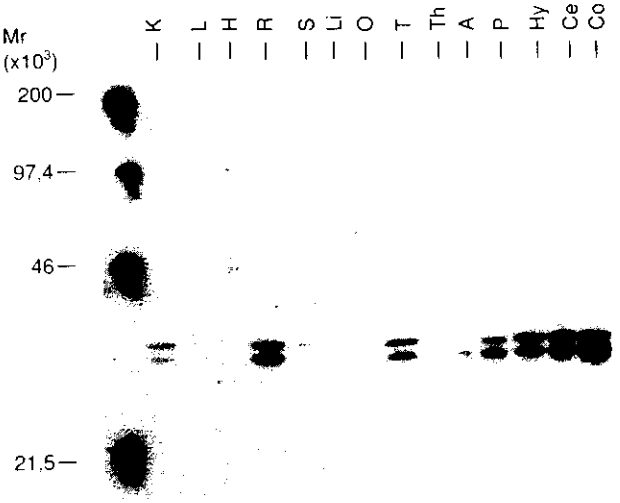


FIG. 9. Immunoblot analysis of the 30- and 33-kD isoforms of the 14-3-3 proteins in various rat tissues. Cytosol was obtained as described in Fig. 8 with the exception that a glass/Teflon homogenizer was used for all tissues other than the pineal. The tissues used were: kidney (K), lung (L), heart (H), retina (R), spleen (S), liver (Li), ovary (O), testis (T), thyroid (Th), adrenal (A), pineal (P), hypothalamus (Hy), cerebellum (Ce), and cerebral cortex (Co). The cytosol (100 μg/lane) was subjected to NaDodSO$_4$-PAGE, electroblotting, and immunodetection as described in the legend to Fig. 8.

1991), and rat (Watanabe *et al.*, 1991) tissues. This is the first report to examine 14-3-3 protein immunoreactivity in the retina, in which relatively high levels were apparent. We also detected a moderate concentration of 14-3-3 protein immunoreactivity in the rat testis, consistent with previous results using human testis (Boston *et al.*, 1982a).

DISCUSSION

The results of this report are relevant to the general topic of 14-3-3 proteins and the specific question of their role in the pineal gland. These aspects will be discussed sequentially.

The new sequence data generated in this study provides further indication of the highly conserved nature of this family of proteins. The alignment in Fig. 5 reveals that the molecules are highly homologous, with several regions that are identical in all the isoforms. These are noted in the consensus line. The longest stretches of high homology (positions 177–195 and 222–241) are predicted to be involved in the formation of beta sheets according to the method of Chou and Fasman (1978). The ε isoform contains three cysteine residues: position 104 is present in all species and isoforms except yeast, position 105 is present only in the ε isoform, and position 201 is present in every species and isoform except human mammary epithelial cell. Additionally, there are two asparagine residues (posi-

tions 185 and 236) present in every species and isoform that are predicted to be glycosylated. However, the results of electrospray mass spectrometric analysis of purified 14-3-3 proteins suggest they are not glycosylated (SD. Howell, B. Green, and A. Aitken, unpublished data).

Comparison of the amino acid sequence of the ϵ isoform to the sequence of plant and vertebrate 14-3-3 proteins indicates that the ϵ isoform is most closely related to plant 14-3-3 proteins (Fig. 5). When comparing the entire molecule, the highest percent identity is with the plant *Arabidopsis thaliana* (74.5%) versus the most closely related mammalian isoform, the human ζ (67.6%). Plants also contain multiple isoforms of the 14-3-3 proteins, each of which shows highest homology with the mammalian ϵ isoform (A. Aitken, unpublished data). Figure 5 also reveals that the sequence of the ϵ isoform is longer than the other mammalian isoforms, yet similar in length to the isoforms cloned from the plants, including spinach (Hirsch *et al.*, 1992), *Oenothera hookeri* (Hirsch *et al.*, 1992), *Arabidopsis thaliana* (Lu *et al.*, 1992), maize (de Vetten *et al.*, 1992), rice (Kidou *et al.*, 1993), and barley (Brandt *et al.*, 1992). Therefore, the sequence of the ϵ isoform is of special interest because it suggests that this might represent the least diverged mammalian isoform.

While the sequences of the 14-3-3 isoforms are highly homologous, there are regions of dissimilarity, for example amino acids 28-47, 76-124, 144-173, and the carboxy-terminal 20 amino acids. The majority of the amino acids in these regions are predicted to be in α-helices; the significance of this is presently unknown.

Another point of general interest relates to the issue of the composition of 14-3-3 dimers and the finding that a partially purified preparation of pineal NAT activity seems to be enriched in only the ϵ and ζ isoforms. In view of the finding that the pineal contains a broad range of 14-3-3 isoforms, copurification of the ϵ and ζ isoforms suggests to us that they exist as an ϵ/ζ heterodimer or as ϵ/ϵ and ζ/ζ homodimers, or as a combination. The existence of homodimers might explain why the 30-kD protein appears to be more abundant in certain tissues (cortex, pineal, adrenal, and retina). The basis and functional significance of specific homodimers and heterodimers is not clear at this time. It seems likely that physiochemical factors determine isoform pairing, and that the different 14-3-3 dimers would have different functions. The potential number of heterodimers is such as to indicate that a very large family of different 14-3-3 dimers might exist.

The final issue of general interest is that our studies on developmental expression and tissue distribution of the 14-3-3 proteins indicate there is significant control of expression of 14-3-3 isoforms. This supports the interpretation that these proteins probably do not represent constitutively expressed housekeeping proteins, but rather are associated with specific functions. Watanabe *et al.* (1993a,b) have demonstrated that the increase or decrease in gene expression of the β, γ, and η isoforms of the 14-3-3 proteins occurs almost synchronously in various rat brain tissues. This is interesting in light of our observation that in the pineal the developmental expression of the ϵ and 30-kD isoforms are inversely related, and suggests that the mechanism by which ϵ expression is regulated in the pineal is distinct from that of the majority of the 30-kD isoforms.

Of specific interest to the role of 14-3-3 proteins in the pineal gland is the observation that a purified preparation of NAT activity is enriched in two isoforms of the 14-3-3 proteins. The 14-3-3 proteins have been implicated in regulating a wide variety of proteins (see Introduction), therefore, it is possible they could regulate NAT activity; this remains to be demonstrated.

Similarly, it will be of interest to determine the functional significance of the developmental changes in pineal 14-3-3 proteins. It is possible that the developmental decrease in the ϵ isoform is linked to the developmental decrease in the expression of photoreceptor features of the mammalian pineal gland (Clabough, 1973; Zimmerman and Tso, 1975), and that the developmental increase in other isoforms of 14-3-3 represents their link with many pineal proteins that appear developmentally during the same period that 30-kD 14-3-3 proteins also appear.

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